



## FGF21 N- and C-termini play different roles in receptor interaction and activation

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### ABSTRACT

**Fibroblast growth factor-21 (FGF21) signaling requires the presence of  $\beta$ -Klotho, a co-receptor with a very short cytoplasmic domain. Here we show that FGF21 binds directly to  $\beta$ -Klotho through its C-terminus. Serial C-terminal truncations of FGF21 weakened or even abrogated its interaction with  $\beta$ -Klotho in a Biacore assay, and led to gradual loss of potency in a luciferase reporter assay but with little effect on maximal response. In contrast, serial N-terminal truncations of FGF21 had no impact on  $\beta$ -Klotho binding. Interestingly, several of them exhibited characteristics of partial agonists with minimal effects on potency. These data demonstrate that the C-terminus of FGF21 is critical for binding to  $\beta$ -Klotho and the N-terminus is critical for fibroblast growth factor receptor (FGFR) activation.**

#### Structured summary:

MINT-6799939: *FGFR1c* (uniprotkb:P11362) binds (MI:0407) to  $\beta$ -Klotho (uniprotkb: Q86Z14) by surface plasmon resonance (MI:0107)

MINT-6799907, MINT-6799922: *FGF21* (uniprotkb: Q9NSA1) binds (MI:0407) to  $\beta$ -Klotho (uniprotkb: Q86Z14) by surface plasmon resonance (MI:0107)

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### 1. Introduction

Fibroblast growth factor-21 (FGF21) belongs to the FGF19 subfamily, which consists of FGF19, 21 and 23 [1]. The FGF19 subfamily members have emerged as potent endocrine hormones involved in the regulation of diverse physiological homeostasis [2–4]. Administration of recombinant FGF21 lowered plasma glucose and insulin levels, reduced hepatic and circulating triglycerides and cholesterol levels, and improved insulin sensitivity, energy expenditure, hepatic steatosis and obesity in a range of insulin resistant animal models [4–6]. Hence, FGF21 has become an attractive therapeutic agent to treat human type 2 diabetes and the associated metabolic syndrome.

Recent progress implied that functional differences between FGF21 and traditional FGFs may arise from differences in their receptor signaling components [7]. Traditional FGFs signal through a family of high affinity receptor tyrosine kinases (FGFR) in the presence of heparin sulfate proteoglycan (HSPG), a low affinity

co-receptor required for FGF/FGFR interactions (see review [8] and references therein). In contrast, the FGF19 subfamily requires distinct Klotho transmembrane proteins as co-receptor for the activation of FGFR signaling [9].  $\alpha$ -Klotho is required for FGF23 to regulate renal phosphate metabolism [10], whereas  $\beta$ -Klotho is required for FGF21 to regulate glucose and lipid metabolism [11] and FGF19 to regulate bile acid metabolism [12]. FGF21 is active in adipocytes and pancreatic  $\beta$ -cells, which express both FGFRs and  $\beta$ -Klotho [4,13,14]. Interestingly, when  $\beta$ -Klotho was transfected in non-responding fibroblasts, FGF21-induced signaling was observed [11,15]. In addition, immunoprecipitation studies indicate that  $\beta$ -Klotho interacts with FGF21 and facilitates the formation of the FGF21– $\beta$ -Klotho–FGFR ternary complex [11,15,16]. Moreover, siRNA knockdown of  $\beta$ -Klotho abolished FGF21-induced signaling and its stimulatory effect on GLUT1 expression and glucose uptake in 3T3-L1 adipocytes [16].

The FGFR isoforms mediating FGF21 signaling are not completely defined. It is known that FGFR4 and the c-isoforms of FGFR1, 2 and 3 are possible candidates [11,15–17]. Immunoprecipitation experiments showed that  $\beta$ -Klotho interacts with FGFR1c and FGFR4 more efficiently than FGFR2c and 3c [11,15,16]. However, in the presence of  $\beta$ -Klotho, FGF21 could signal in BaF3 cells expressing either FGFR1c or 3c, but not 2c or FGFR4 [18]

Abbreviations: FGF, fibroblast growth factor; ERK, extracellular signal-regulated kinase; HSPG, heparin sulfate proteoglycan

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and in L6 myoblasts expressing FGFR1c, 2c or 3c, but not FGFR4 [16]. Therefore, the c-isoforms of FGFRs rather than FGFR4 might be physiologically relevant receptors for FGF21 and mediate FGF21 activity through their respective tissue expression patterns. Independently, FGFR4 has been shown to be a specific receptor for FGF19 [19].

Despite the progress made in the identification of FGF21 receptor signaling components, the nature of the interactions between FGF21, FGFR and  $\beta$ -Klotho is not completely understood. Structure-based modeling and sequence alignment suggest that FGF21 has a typical 120 aa  $\beta$ -trefoil core structure like the traditional FGFs, but its N- and C-terminal sequences are significantly different from those of other FGFs ([9], and unpublished data). Here we sought to use FGF21 N- and C-terminal truncations to identify the regions of FGF21 responsible for FGFR and  $\beta$ -Klotho interactions and thereby better understand these regions' roles in FGF21 receptor activation.

## 2. Materials and methods

### 2.1. FGF21, $\beta$ -Klotho and FGFR1c protein and plasmids

Full-length and truncated human FGF21 proteins were expressed in an *Escherichia coli* strain using a proprietary vector. The expression was primarily found in inclusion bodies. The recovery of oxidized proteins involved the dissolution of the inclusion bodies and refolding of the soluble reduced protein. Briefly, *E. coli* cells were resuspended in water and lysed with a 110S Microfluidizer. The inclusion bodies were dissolved with a buffer of 8 M guanidine, 50 mM Tris, pH 8.5, and 10 mM dithiothreitol. The solution was subsequently added to a refolding solution to achieve a final concentration of 1 mg/ml. The refolded material was further purified by anion exchange chromatography, followed by hydrophobic interaction chromatography. All deletion mutants were tested by Western blotting and reverse-phase HPLC to ensure protein integrity. No deletion resulted in significant structural changes as judged by spectrum photometric measurement.

The extracellular domain of human  $\beta$ -Klotho (1–992) fused with 6xHis tag at the C-terminus was cloned into the pTT14 expression vector. The extracellular domain of  $\beta$ -Klotho protein was purified from a 293F stable cell line using Talon IMAC resin- $\text{Co}^{2+}$  (Clontech) according to the manufacturer's protocol, and was further purified with CHT Type I 40  $\mu\text{m}$  column (Bio-Rad). The recombinant FGFR1c-Fc fusion protein with the extracellular domain of FGFR1c at the N-terminus was generated internally with similar method as the protein available commercially from R&D systems.

### 2.2. ELK luciferase assay

ELK luciferase assay was performed in 293T cells that were stably transfected with human  $\beta$ -Klotho and reporter constructs containing 5xUAS luciferase and GAL4 DNA-binding domain (DBD) fused to ELK1. In this system, luciferase activity is regulated by the endogenous phosphorylated extracellular signal-regulated kinase (ERK). The 293T stable cells were seeded at  $1 \times 10^5$  cells/well on 96-well plates. On the next day, FGF21 proteins were added to the media. The plates were incubated for 6 h. Cells were then lysed to measure luciferase activity using the Bright-Glo luciferase assay system (Promega).

### 2.3. Biacore binding studies

Binding of FGF21 and its mutants to human  $\beta$ -Klotho was tested in a Biacore solution equilibrium binding assay. Briefly, NeutrAvidin (Pierce) was immobilized on a CM5 chip using amine coupling

reagents (GE Healthcare). Biotinylated FGF21 was captured on the second flow cell to 800RU. The biotinylated FGF21 has similar activity as native FGF21 in the luciferase assay and the Biacore binding assay (data not shown). Each FGF21 mutant was incubated at three different concentrations (2  $\mu\text{M}$ , 200 nM and 20 nM) with 10 nM human  $\beta$ -Klotho in a PBS buffer containing 0.1 mg/ml BSA and 0.005% P20 for 1 h at room temperature. The pre-incubated mixtures were injected over the biotin-FGF21 surface to measure the binding of free  $\beta$ -Klotho. The signal obtained with no FGF21 mutant in the pre-incubation represents 100%  $\beta$ -Klotho binding and serves as control. Decreased signal with increasing concentrations of a given FGF21 mutant in the pre-incubation indicates that it blocks  $\beta$ -Klotho binding to the immobilized biotin-FGF21 and therefore, retains ability to bind to  $\beta$ -Klotho.

Binding of human  $\beta$ -Klotho to FGFR1c-Fc was also tested in the Biacore assay. Briefly, FGFR1c-Fc was immobilized on the second flow cell of a CM5 chip using amine coupling to  $\sim 10,000$  RU. The first flow cell was used as a background control. Twenty nanomolars of human  $\beta$ -Klotho diluted in PBS plus 0.1 mg/ml BSA and 0.005% surfactant P20 was injected over the FGFR1c-Fc surface for 5 min at 10  $\mu\text{l}/\text{min}$ .

### 2.4. Statistical analysis

All data are means  $\pm$  standard deviation (S.D.). Values were compared using Student's paired *t*-test.  $P < 0.05$  was considered as statistically significant.

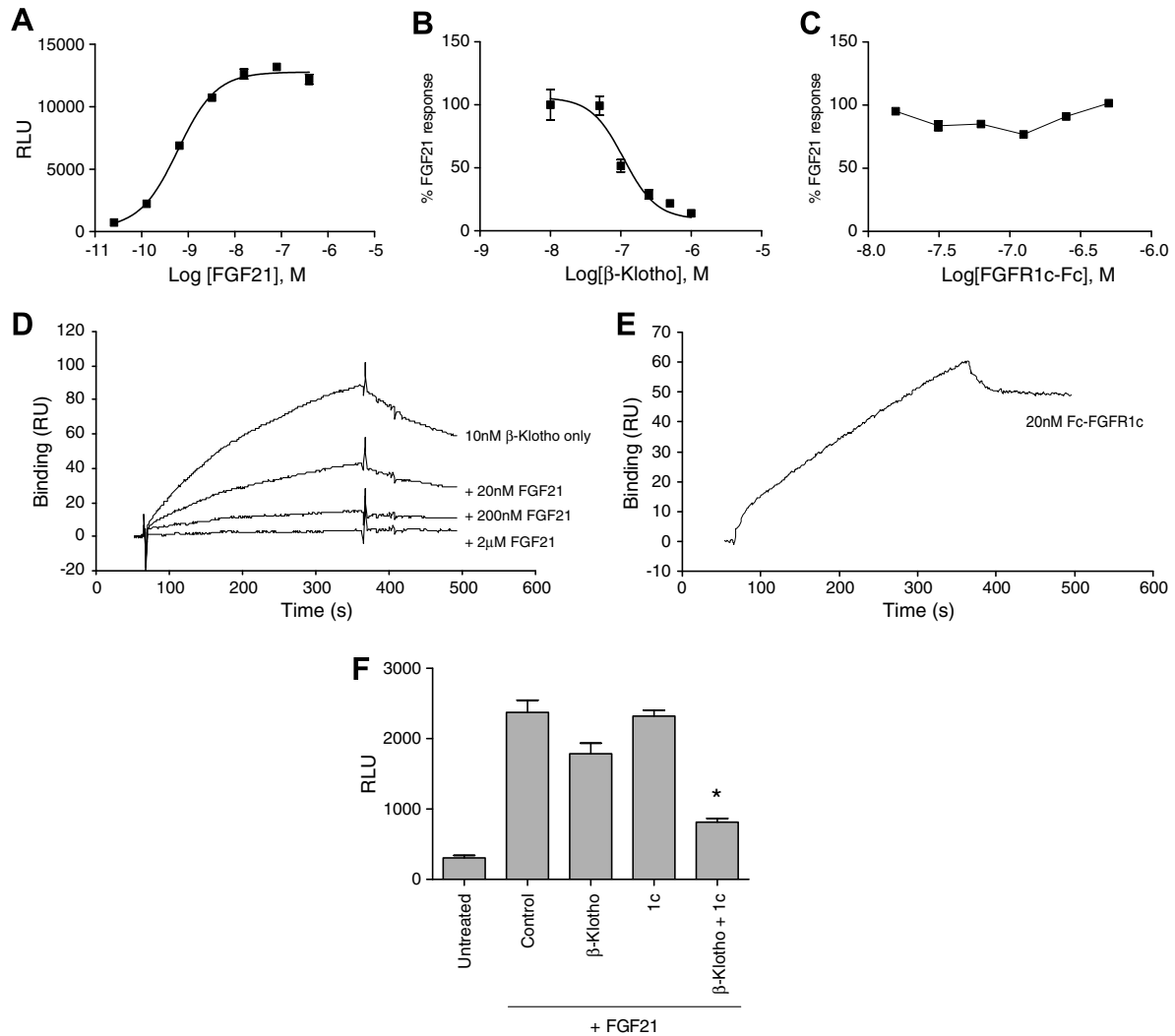
## 3. Results

### 3.1. Direct interaction of FGF21 with $\beta$ -Klotho

We developed a luciferase reporter assay system in 293T cells that stably express human  $\beta$ -Klotho and reporter constructs. Then, we measured and determined that these cells express multiple FGFRs including FGFR1c endogenously (data not shown). When FGF21 was tested in this cell line, ERK phosphorylation was induced leading to elevated luciferase activity ( $\text{EC}_{50} = 0.5$  nM, Fig. 1A). To test whether FGF21 could directly bind to  $\beta$ -Klotho or FGFR, we set up a competition assay using the same reporter system. FGFR1c was selected as the FGFR since it is the most validated receptor for the FGF21 signaling [11,15–17]. Either soluble  $\beta$ -Klotho alone or FGFR1c-Fc alone did not affect luciferase activity (data not shown). However, soluble  $\beta$ -Klotho decreased FGF21-induced luciferase activity in a dose-dependent manner ( $\text{IC}_{50} \sim 110$  nM, Fig. 1B), suggesting that the soluble  $\beta$ -Klotho may directly bind to FGF21 and sequester it from acting on the cells. In contrast, FGFR1c-Fc did not inhibit FGF21 signaling (Fig. 1C), indicating that no interaction between FGFR1c and FGF21 may have occurred. Interaction between  $\beta$ -Klotho and FGF21 was further assessed in a direct binding study using Biacore. Interaction between FGF21 and  $\beta$ -Klotho was detected (Fig. 1D). Soluble FGF21 inhibited  $\beta$ -Klotho binding to immobilized FGF21 with an  $\text{IC}_{50}$  around 18.6 nM (Fig. 1D). The estimated  $K_d$  ( $k_{\text{off}}/k_{\text{on}}$ ) value is approximately 15 nM. No direct binding between FGFR1c and FGF21 was detected (data not shown), a result that is consistent with the finding from the luciferase assay where soluble FGFR1c did not inhibit FGF21 signaling (Fig. 1B). Other FGFRs such as FGFR2c, FGFR3c and FGFR4 were also tested in the Biacore assay and no direct binding between these FGFRs and FGF21 was detected (data not shown).

### 3.2. Synergism of FGFR1c and $\beta$ -Klotho in FGF21 signaling

Several immunoprecipitation experiments have shown that  $\beta$ -Klotho interacts with FGFR1c [11,15,16]. Surprisingly,  $\beta$ -Klotho/



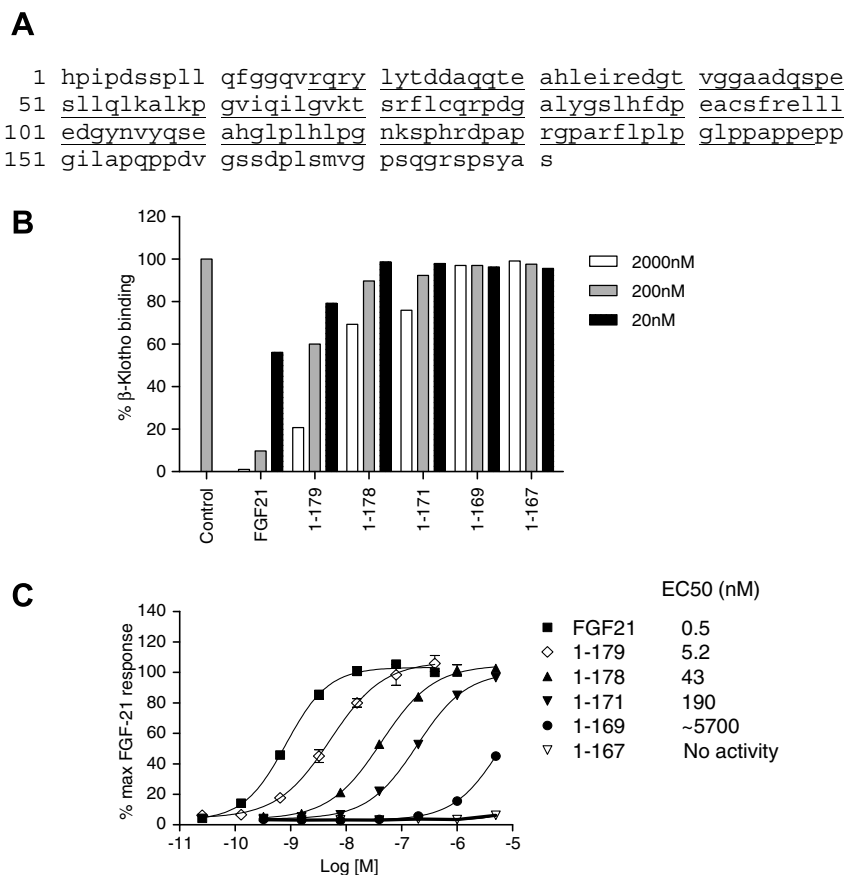
**Fig. 1.** Interaction of FGF21 with  $\beta$ -Klotho. (A) FGF21 stimulates luciferase activity in a dose-dependent manner as measured in a 293T reporter cell line stably expressing human  $\beta$ -Klotho and reporter genes ( $EC_{50} \sim 0.5$  nM). (B) Soluble  $\beta$ -Klotho inhibits FGF21 activity in the same reporter assay. Different amounts of soluble  $\beta$ -Klotho were incubated with  $EC_{50}$  amount of FGF21 (5 nM) for 15 min on ice before being added to the cells. The  $IC_{50}$  for  $\beta$ -Klotho inhibition is  $\sim 110$  nM. (C) Different amounts of soluble FGFR1c-Fc were incubated with 5 nM FGF21 before being added to the cells. Soluble FGFR1c-Fc did not inhibit FGF21 activity in the reporter assay. (D) FGF21 directly binds to  $\beta$ -Klotho in a Biacore assay. Shown is the binding sensorgram of  $\beta$ -Klotho to immobilized biotin-FGF21. Biotin-FGF21 was captured on a CM5 chip coated with NeutrAvidin. Human  $\beta$ -Klotho (10 nM) was pre-incubated with different amounts of FGF21 for 1 h before the mixture was injected over the biotin-FGF21 surface.  $\beta$ -Klotho bound to the chip surface was quantitated. (E) FGFR1c-Fc directly binds to  $\beta$ -Klotho in a Biacore assay. Shown is the binding sensorgram of 20 nM  $\beta$ -Klotho to immobilized FGFR1c-Fc. (F) Synergism between soluble FGFR1c-Fc and soluble  $\beta$ -Klotho in inhibiting FGF21 from activation was observed in the reporter assay. FGF21 (5 nM), soluble  $\beta$ -Klotho (125 nM), and soluble FGFR1c-Fc (500 nM) were pre-incubated for 15 min before being added to the cells. The inhibition by the mixture of FGFR1c-Fc and  $\beta$ -Klotho was significantly greater than  $\beta$ -Klotho alone (\* $P < 0.05$  compared with  $\beta$ -Klotho alone).

FGFR1c interaction was remarkably weak in our Biacore assay (Fig. 1E). We estimated that the  $K_d$  for  $\beta$ -Klotho/FGFR1c interaction was greater than 100 nM.

Since both  $\beta$ -Klotho and FGFR1c are required for FGF21 activity, we next tried to determine if both receptors could bind to FGF21 cooperatively. A suboptimal dose of soluble  $\beta$ -Klotho (125 nM), which could only partially inhibit FGF21 activity (Fig. 1F), was pre-incubated with FGFR1c and FGF21. The mixture was then added to the cells. Interestingly, when FGFR1c and  $\beta$ -Klotho were added together, FGF21 activity was suppressed to a greater degree than  $\beta$ -Klotho alone (Fig. 1F). This effect appeared to be synergistic and was seen in the presence of a broad range of  $\beta$ -Klotho concentrations (data not shown). Thus,  $\beta$ -Klotho and FGFR1c may form a complex with a higher affinity for FGF21 than  $\beta$ -Klotho alone. In contrast, the synergistic effect of  $\beta$ -Klotho/FGFR1c was not seen in a similar experiment when soluble FGFR4 and  $\beta$ -Klotho were added together (data not shown).

### 3.3. FGF21 binds to $\beta$ -Klotho through its C-terminal region and the interaction regulates FGF21 potency

In order to identify the regions of FGF21 that interact with its receptors, we generated a series of FGF21 deletion mutants. We first focused on the C-terminus as this region of FGF23, a close family member of FGF21, is critical for the interaction with  $\alpha$ -Klotho [9]. As shown in Fig. 2B, serial C-terminal deletions of FGF21 resulted in gradual loss of its binding to  $\beta$ -Klotho, and 14 aa deletion at the C-terminus resulted in complete loss of  $\beta$ -Klotho binding. Interestingly, serial C-terminal deletions of FGF21 also resulted in gradual loss in potency when tested in the reporter assay (Fig. 2C), and the reporter activity was completely lost when the C-terminal 14 aa were removed (Fig. 2C). Despite the loss of potency, it appears that most C-terminal deletion mutants maintained full efficacy (maximal response) (Fig. 2C). The affinity of FGF21 C-terminal deletion mutants for  $\beta$ -Klotho matched the po-



**Fig. 2.** C-terminal FGF21 is critical for  $\beta$ -Klotho binding and the binding to  $\beta$ -Klotho determines FGF21's potency in the reporter assay. (A). Shown is the amino acid sequence of human FGF21. Signal peptide sequence is omitted. Underlined is the predicted  $\beta$ -trefoil core domain of FGF21 [9]. (B) Biacore data with FGF21 C-terminal deletion mutants. As described in Fig. 1D, different amounts of FGF21 deletion mutants were incubated with 10 nM  $\beta$ -Klotho for 1 h before the mixture was injected over the biotin-FGF21 surface. Free  $\beta$ -Klotho was used as a control to demonstrate maximal binding. Concentrations of FGF21 constructs used are: white bar, 2  $\mu$ M; gray bar, 200 nM; black bar, 20 nM. (C) The luciferase reporter assay of the selected FGF21 C-terminal deletion mutants in the 293T stable cell line. These data have been repeated multiple times with triplicate in each experiment.

tency in the reporter assay, suggesting that  $\beta$ -Klotho is likely an anchor that brings FGF21 to the close proximity of FGFR to induce reporter-mediated signaling.

#### 3.4. The N-terminal region of FGF21 is important for FGF21 activation

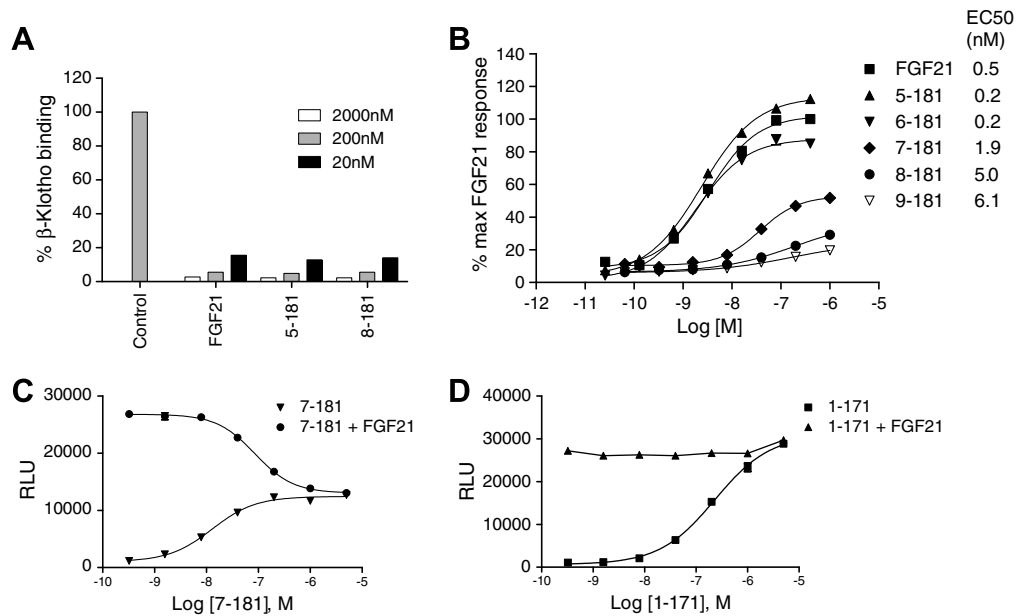
To understand the role of N-terminal FGF21 in receptor interaction, we generated N-terminal serial deletion mutants of FGF21. In contrast to the C-terminal deletions, all the N-terminal deletion mutants of FGF21 retained their binding to  $\beta$ -Klotho in the Biacore assay (Fig. 3A). In the reporter assay, a slight increase in efficacy was observed when the first 4 aa were removed at the N-terminus, suggesting that these 4 aa are not required for FGF21 activity (Fig. 3B). When additional N-terminal amino acids were deleted, gradual decreases in efficacy (maximal response) were noticed (Fig. 3B). FGF21 signaling was almost completely lost after removal of 8 aa at the N-terminus (9–181, Fig. 3B). Interestingly, all N-terminal deletions had very little effect on potency (EC<sub>50</sub>) (Fig. 3B).

The reporter activation curves for the N-terminal deletion mutants exhibited partial agonist characteristics (Fig. 3B). To further demonstrate partial agonist properties of these mutants, we assessed the effect of different concentrations of 7–181 on the reporter activity in the presence of full-length FGF21 at 5 nM concentration (~EC<sub>90</sub> dose). Interestingly, 7–181 inhibited the full-length FGF21 signaling in a dose-dependent manner, with the maximal inhibitory response corresponding to the maximal

activation obtained with 7–181 alone (Fig. 3C). These data confirmed the notion that 7–181 is a partial agonist as it can inhibit the activity by the full agonist. Also, it is fair to assume that the partial agonist effect was not caused by changes in  $\beta$ -Klotho binding since the N-terminal deletion mutants retained their ability to bind to  $\beta$ -Klotho (Fig. 3A). In contrast to the N-terminal deletion mutants, the C-terminal deletion mutant 1–171 did not suppress FGF21 signaling (Fig. 3D), suggesting that these mutants are simply weak agonists. Interestingly, most C-terminal deletion mutants acted as full agonists despite their reduced potency and binding to  $\beta$ -Klotho (Fig. 2B and C). Altogether, these data suggest that the N- and C-termini of FGF21 likely play very different roles in  $\beta$ -Klotho binding and FGFR activation.

#### 4. Discussion

FGF21, along with FGF19 and FGF23, belongs to an unusual subfamily of the FGF superfamily. Traditional FGFs have no co-receptors, and they directly induce FGFR dimerization, a common activation mechanism for most FGFs [20]. However, the FGF19 subfamily members require the Klotho proteins as co-receptors in receptor activation. FGF21 signaling requires  $\beta$ -Klotho, which has only a few amino acids in the intracellular region [14]. Hence, FGF21 signaling seems to be solely conducted through FGFR. How FGF21 interacts with FGFR or  $\beta$ -Klotho is not clear. In this paper, we showed that the FGF21 C-terminus is critical for  $\beta$ -Klotho



**Fig. 3.** N-terminal FGF21 deletions resulted in partial agonist effect and have no impact on  $\beta$ -Klotho interaction. (A) The N-terminal deletion mutants were assessed for their ability to block  $\beta$ -Klotho binding to FGF21. Experimental procedure is same as described in Figs. 1D and 2A. Concentrations of FGF21 constructs used were: white bar, 2  $\mu$ M; gray bar, 200 nM; black bar, 20 nM. (B) The luciferase reporter assay of the selected FGF21 N-terminal deletion mutants in the 293T stable cell line. These data have been repeated multiple times with triplicate in each experiment. The EC<sub>50</sub> data shown are the average data of multiple experiments. 8-181 and 9-181 have been repeated at higher doses to obtain more accurate EC<sub>50</sub> value. (C) 7-181 inhibited the activity of full-length FGF21 in the reporter assay. 7-181 was pre-incubated with 5 nM full-length FGF21 for 15 min at room temperature before added to the 293T stable cells for reporter assay. (D) 1-171 did not compete with full-length FGF21 in the reporter assay.

binding and the N-terminus for the efficacy of FGFR activation. Deleting the C-terminus resulted in loss of potency in the luciferase assay, whereas deleting the N-terminus led to reduced maximal response, or partial agonist effect.

Structure modeling suggests that FGF21 has a typical 120 aa  $\beta$ -trefoil core structure as seen in the traditional FGFs, but its N- and C-terminal sequences are significantly different from those in other FGFs [9]. Our study demonstrated that the 14 aa at C-terminus were necessary for  $\beta$ -Klotho binding and overall FGF21 activity. The relative importance of the individual amino acids in  $\beta$ -Klotho binding could be determined using alanine-scanning in the future. Further, C-terminal deletions reduced the affinity for  $\beta$ -Klotho and the potency in the reporter assay, suggesting that  $\beta$ -Klotho binding is critical to obtain FGF21 potency. Interestingly, a naturally occurring form of FGF23 that lacks the C-terminal 73 aa [21] failed to bind to  $\alpha$ -Klotho and was inactive in signaling [9]. This finding is consistent with our data with the FGF21 C-terminus and together they suggest that the C-terminus of the FGF19 subfamily members may be critical for co-receptor binding. Also, our data further imply that  $\beta$ -Klotho binding may be a prerequisite for FGF21 activation. Unlike traditional FGFs, FGF21 acts independently of heparin [9]. This allows FGF21 to escape cell membranes which are abundant with HSPG and function as an endocrine factor [9]. Our data is consistent with the endocrine nature of FGF21 in using  $\beta$ -Klotho as an anchor to avoid misfiring. It is the combination of tissue-specific expression of  $\beta$ -Klotho and FGFR subtypes that define tissue selectivity of FGF21 [16].

In contrast, the N-terminus of FGF21 does not play a role in  $\beta$ -Klotho binding. The N-terminal deletions of FGF21 retained binding to  $\beta$ -Klotho in the Biacore assay, and had very little impact on FGF21 potency in the luciferase assay. Instead, decreases in efficacy (maximal response) were observed with these mutants. Furthermore, the efficacy was drastically reduced with 6 aa deletion at the N-terminus and completely lost with 8 aa deletion. This decreased efficacy suggests that these deletion mutants behave as

partial agonists. Indeed, in the reporter activation assay, 7-181 inhibited the activity of the full-length FGF21, a typical characteristic of a partial agonist. Although the N-terminus of FGF21 is important for efficacy, it is not clear what role this region plays in FGFR activation. One study on FGF1–FGFR3c structure revealed extensive interactions between the  $\beta$ C'– $\beta$ E loop (within the D2 domain of FGFR3c) and the N-terminal region of FGF1 [22], underscoring the importance of the N-terminal region in conferring receptor binding affinity and promiscuity. However, to date, no evidence of direct interaction between FGF21 and FGFR has been reported. We were not able to demonstrate direct interaction between FGF21 and FGFR1c in the Biacore assay (data not shown). Modeling studies suggest that the FGF19 subfamily members may have inherently low affinity for their FGFRs [9]. The potential participation of FGF21 N-terminus in receptor interaction and activation will require further investigation.

While this manuscript was prepared, a different group showed similar data on FGF21 N- and C-termini deletion mutants [23]. It confirmed our findings of the role of the C-termini in  $\beta$ -Klotho binding. However, this group did not identify the partial agonist effect of N-terminal deletion mutants, probably due to relatively low sensitivity of the assays in their study.

Our data suggest that  $\beta$ -Klotho may play a central role by binding to and recruiting FGF21 to FGFR to induce activation. When soluble FGFR1c and  $\beta$ -Klotho were incubated with FGF21, they exhibited greater inhibitory effect on FGF21 activity than  $\beta$ -Klotho alone (Fig. 1E). Even though specific interaction between FGFR1c and  $\beta$ -Klotho has been detected in immunoprecipitation experiments [11,15,16], the binding affinity of FGFR1c for  $\beta$ -Klotho is low (K<sub>d</sub> > 100 nM, Fig. 1E). In addition, the relative binding affinity of FGF21 for  $\beta$ -Klotho is also weak (K<sub>d</sub> ~ 15 nM, Fig. 1D). Given the high potency of FGF21 in the reporter activation assay (EC<sub>50</sub> ~ 0.5 nM) and the data mentioned above, FGF21,  $\beta$ -Klotho and FGFR1c may form a ternary complex in a highly coordinated manner.

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